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Rab7 Prevents Growth Factor-Independent Survival by Inhibiting Cell-Autonomous Nutrient Transporter Expression

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Summary

Growth factor withdrawal results in the endocytosis and degradation of transporter proteins for glucose and amino acids. Here, we show that this process is under the active control of the small GTPase Rab7. In the presence of growth factor, Rab7 inhibition had no effect on nutrient transporter expression. In growth factor-deprived cells, however, blocking Rab7 function prevented the clearance of glucose and amino acid transporter proteins from the cell surface. When Rab7 was inhibited, growth factor deprived cells maintained their mitochondrial membrane potential and displayed prolonged, growth factor-independent, nutrient-dependent cell survival. Thus, Rab7 functions as a proapoptotic protein by limiting cell-autonomous nutrient uptake. Consistent with this, dominant-negative Rab7 cooperated with E1A to promote the transformation of *p53*$^{-/-}$ mouse embryonic fibroblasts (MEFs). These results suggest that proteins that limit nutrient transporter expression function to prevent cell-autonomous growth and survival.

Introduction

A requirement for multicellularity is that cellular growth is constrained within the limits of tissue homeostasis. Unlike unicellular eukaryotes, the proliferation of mammalian cells is not determined by the availability of extracellular nutrients. Rather, mammalian cells are constantly supplied with nutrients and cell growth is controlled through cellular dependence on extrinsic factors produced by other cells (Conlon and Raff, 1999; Raff, 1992). These growth factors provide signals that allow cells to survive, divide, and accumulate mass. When growth factors become limiting, cells withdraw from the cell cycle, atrophy, and undergo apoptosis via an intrinsic cell death pathway initiated by mitochondria. Thus, growth factor dependence represents an important obstacle to neoplastic transformation. Tumor cells must acquire mutations that allow them to avoid apoptosis and to proliferate in the absence of extrinsic signals.

When cells are withdrawn from growth factors, the rates of glucose and amino acid uptake decline rapidly (Edinger and Thompson, 2002; Kan et al., 1994; Vander Heiden et al., 2001; Whetton et al., 1984). In addition, transporters for iron (transferrin receptor) and cholesterol (LDL receptor) are also rapidly lost from the surface of growth factor-deprived cells (Edinger and Thompson, 2002). These observations suggest that growth factors may directly regulate the uptake of extracellular nutrients by controlling nutrient transporter expression. Although this decline in nutrient uptake could result from a decrease in cellular demand due to the loss of growth factor stimulation of cell growth, growth factor withdrawn cells exhibit characteristics of starvation, such as cellular atrophy and autophagic proteolysis. This growth factor withdrawal-induced state of pseudostarvation produced by nutrient transporter downregulation may provide the trigger for subsequent apoptosis by affecting cellular bioenergetics. Alterations in mitochondrial metabolism, reflected by decreases in the mitochondrial membrane potential, precede the changes in mitochondrial physiology that ultimately result in the release of proapoptotic mediators (Vander Heiden et al., 1997, 1999). Activated forms of the protooncogene *Akt* support growth factor-independent survival and preserve the mitochondrial membrane potential through a mechanism that depends on the availability of extracellular nutrients and on the continued expression of nutrient transporters on the cell surface (Cantley and Neel, 1999; Edinger and Thompson, 2002; Gottlob et al., 2001; Plas et al., 2001). These results suggest that the antiapoptotic potential of Akt is closely tied to its ability to promote growth factor-independent nutrient uptake.

Following endocytosis, cell surface proteins enter either a degradative or a recycling pathway (Mellman, 1996). Proteins targeted to the degradative pathway transit through the multivesicular body and ultimately enter the lysosome where they are subject to proteolytic degradation. It has been suggested that proteins that are not explicitly tagged for degradation enter a default, recycling pathway and are reexpressed on the cell surface. One of the proteins involved in this sorting process is the small GTPase Rab7. Rab7 catalyzes late endosome-lysosome (heterotypic) and lysosome-lysosome (homotypic) membrane fusion reactions through a mechanism that is incompletely understood (Bucci et al., 2000; Feng et al., 1995). Thus, Rab7 has a positive role in the degradation of plasma membrane proteins.

If nutrient transporter downregulation sets up a state of pseudostarvation that ultimately results in the initiation of apoptosis, then interfering with the internalization and degradation of these transporter proteins should protect cells from growth factor withdrawal-induced cell death. To test whether the rapid atrophy, decline in cellular bioenergetics, and programmed cell death associated with growth factor withdrawal was directly caused by or merely correlated with the loss of access to extracellular nutrients due to transporter downregulation, we prevented the degradation of nutrient transporters by interfering with the function of Rab7. Consistent with the hypothesis that nutrient transporter downregulation plays a causative role in these processes, the inhibition of Rab7 function resulted in a nutrient-replete cellular phenotype despite growth factor withdrawal. In addition, cells in which nutrient transporter expression was supported by blocking Rab7 function displayed long-term resistance to growth factor withdrawal-induced cell death but were as sensitive as control cells

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to apoptosis induced by nutrient limitation. Interfering with the function of the downstream protein RILP also protected cells from growth factor withdrawal-induced cell death, suggesting that other proteins in this pathway may also regulate growth factor withdrawal-induced apoptosis. Furthermore, disrupting Rab7 function facilitated the transformation of *p53*$^{-/-}$ MEFs by E1A. These results suggest that Rab7 contributes to the control of cellular growth by limiting the ability of cells to take up extracellular nutrients in a cell-autonomous fashion.

Results

Nutrient Transporter Expression Is Maintained following Growth Factor Withdrawal When Rab7 Function Is Inhibited

Dominant-negative forms of many GTPases can be produced through point mutations in the GTP binding domain (Feng et al., 1995). The point mutation T22N in the GTP binding domain of Rab7 produces such a dominant-negative protein (Feng et al., 1995; Vitelli et al., 1997). When Rab7 T22N is expressed in fibroblasts, heterotypic and homotypic lysosomal fusion reactions are inhibited and plasma membrane proteins are recycled to the cell surface rather than degraded in lysosomes (Bucci et al., 2000; Feng et al., 1995; Vitelli et al., 1997). We stably expressed Rab7 T22N in FL5.12 cells, a murine hematopoetic cell line. The FL5.12 cell line was selected for these studies because it is dependent on a defined growth factor, IL-3, for growth and survival. For ease in detection and screening, we expressed Rab7 T22N as an N-terminal EGFP fusion protein (Bucci et al., 2000).

FL5.12 cell clones stably expressing Rab7 T22N were screened by flow cytometry for EGFP expression. Two lines expressing high levels of EGFP were labeled T22N 1 and T22N 2 and used for further analyses. These lines were screened by Western blot to evaluate Rab7 T22N expression level (Figure 1A). EGFP-Rab7 T22N was expressed in both lines at approximately the same level as endogenous Rab7. The inhibition of Rab7 function had no effect on cell growth under normal culture conditions consistent with the observation that the cloning efficiency for Rab7 T22N and empty vector was equivalent (data not shown). To confirm that this level of Rab7 T22N was sufficient to interfere with the function of endogenous Rab7, lysosomal morphology was evaluated (Figure 1B and data not shown). Lysosomal fragmentation was observed in T22N 1 and T22N 2, confirming that Rab7 function was inhibited in these clones.

The Rab7 dependence of growth factor withdrawal-induced degradation of 4F2hc and Glut1 was evaluated. The amino acid transporter-associated protein 4F2hc was selected for these studies as few mammalian amino acid transporters have been molecularly cloned, and antibodies to these proteins are not readily available (Palacin et al., 1998). 4F2hc forms a heterodimeric complex with a variety of light chains, directing these proteins to the cell surface where they transport cationic amino acids (Deves and Boyd, 2000; Palacin et al., 1998). Consistent with its role in amino acid uptake, 4F2hc is an early activation marker in lymphocytes and is highly expressed on a variety of tumor cells. To perform immunofluorescence studies on cells withdrawn from IL-3,

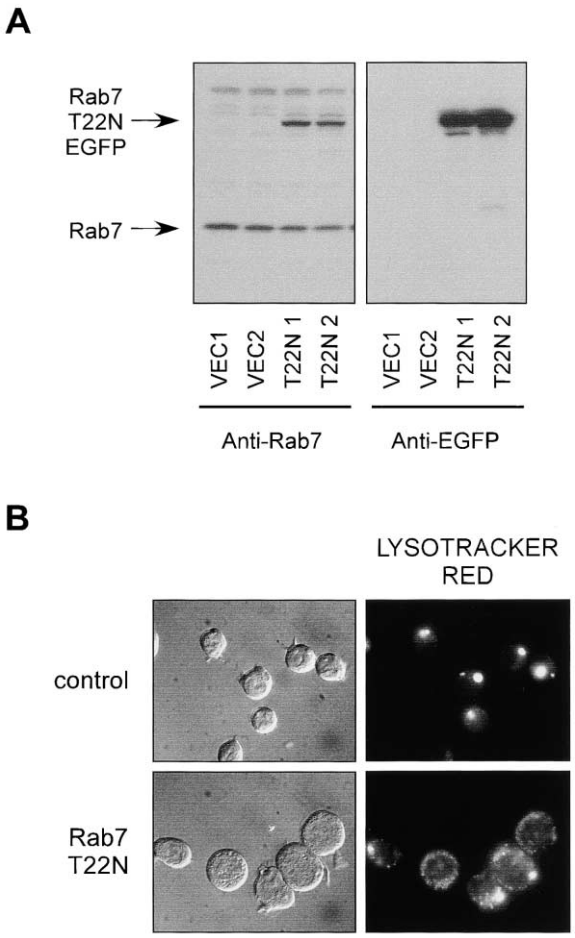


Figure 1. Dominant-Negative Rab7 Can Be Stably Expressed in Growth Factor-Dependent Cells

(A) Rab7 T22N was stably expressed in FL5.12 cells as an N-terminal EGFP fusion protein. Two clones expressing Rab7 T22N at a similar level to the endogenous protein, T22N 1 and T22N 2, were selected for further studies.

(B) FL5.12 cells stably expressing Rab7 T22N or empty vector were stained with LysoTracker Red to evaluate lysosomal morphology. Apparent differences in cell diameter reflect the relative extent of cell flattening by the coverslip rather than true differences in cell size.

Bcl-X_L was coexpressed with Rab7 T22N. Bcl-X_L prevents cell death upon growth factor withdrawal but does not interfere with nutrient transporter downregulation (Plas et al., 2001; Rathmell et al., 2000; Vander Heiden et al., 2001; and A.L.E., R.M.C., and C.B.T., unpublished data). Thus, transporter turnover can be examined in the absence of the confounding effects of cell death through the coexpression of Bcl-X_L.

In control cells maintained in IL-3, 4F2hc was found primarily on the cell surface (Figure 2A). When IL-3 was withdrawn from these cells, bright intracellular spots of 4F2hc staining developed. Interfering with Rab7 function had no effect on 4F2hc expression in the presence of growth factor. However, when growth factor was withdrawn, focal intracellular staining for 4F2hc did not develop in Rab7 T22N-expressing cells. Rather, many small spots of 4F2hc staining were apparent. These results are consistent with an intact endocytic pathway

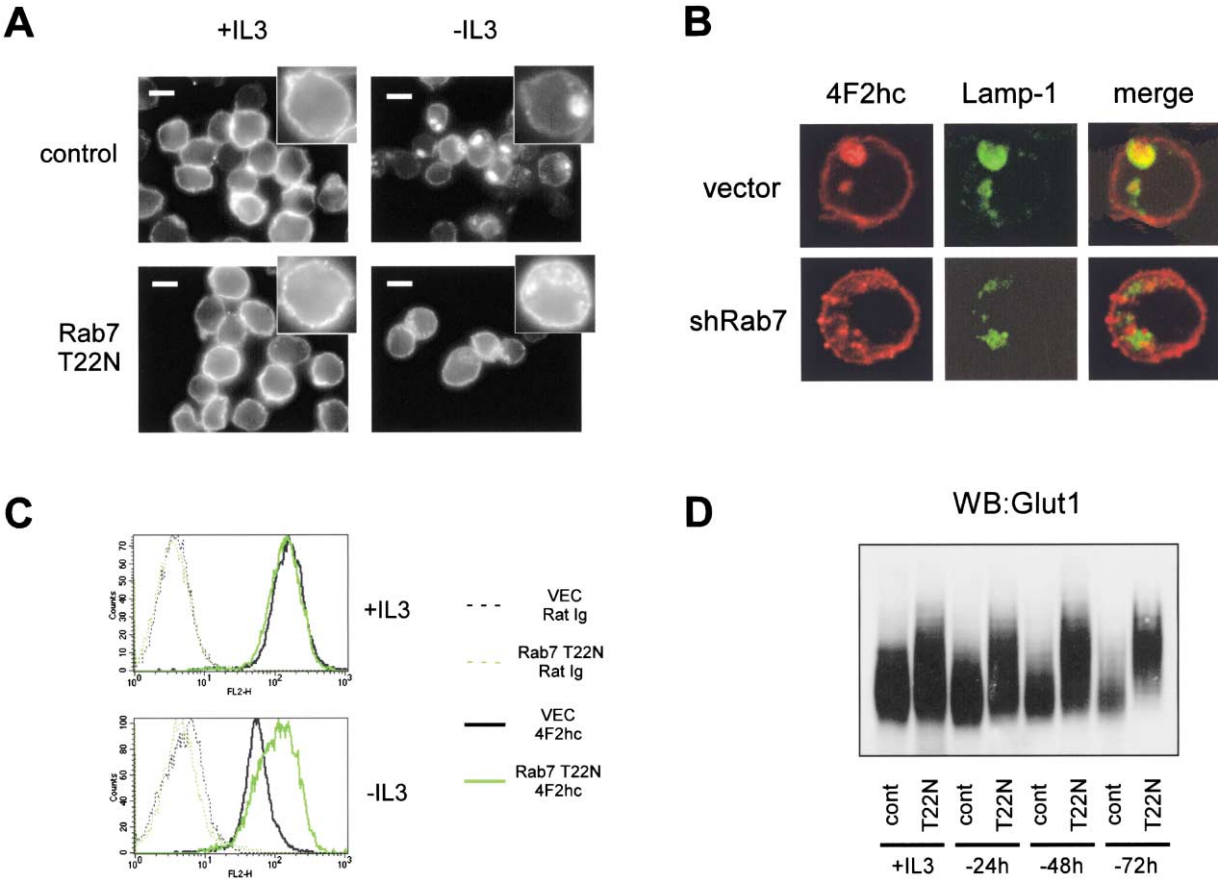


Figure 2. Rab7 Function Is Required for Degradation of the Amino Acid Transport-Associated Protein 4F2hc and Glut1 in Response to Growth Factor Withdrawal

(A) Control cells expressing Bcl-X_L or cells also expressing Rab7 T22N were maintained in the presence or absence of IL-3 as indicated for 24 hr then fixed and stained for 4F2hc expression. Scale bar represents 10 μm. Insets show a single cell magnified to more clearly visualize the staining pattern.

(B) FL5.12 cells stably expressing empty vector or a short hairpin directed against Rab7 (shRab7) were withdrawn from IL-3 for 14 hr and 4F2hc and Lamp-1 colocalization evaluated by confocal microscopy.

(C) Cells were prepared as in (A), stained for surface 4F2hc expression at 48 hr, and analyzed by flow cytometry. Analysis was restricted to live cells. One of three similar experiments is shown.

(D) To investigate the changes in Glut1 expression in response to IL-3 withdrawal, control cells expressing Bcl-X_L or cells also expressing Rab7 T22N were maintained in the presence or absence of IL-3 for the indicated intervals, lysed, and Glut1 protein levels determined by Western blot. Ten micrograms of total protein isolated from purified live cells were loaded in each lane. Based on densitometry, there was a nearly 2-fold difference in the degree of decline in Glut1 levels between control and T22N-expressing cells. In addition, the electrophoretic mobility of the remaining Glut1 was distinctly different in the two cell lines. One of three similar experiments is shown.

but a defect in endosome-lysosome fusion as has been reported in other cell types expressing Rab7 T22N.

To confirm that interfering with Rab7 function decreased the trafficking of 4F2hc to lysosomes upon IL-3 withdrawal, levels of endogenous Rab7 were reduced by RNAi, and the localization of 4F2hc and the lysosomal membrane protein Lamp-1 were evaluated in IL-3 withdrawn FL5.12 cells by confocal microscopy (Figure 2B). While intracellular 4F2hc and Lamp-1 colocalized in control cells deprived of growth factor, 4F2hc and Lamp-1 staining did not overlap in IL-3 withdrawn cells expressing reduced levels of Rab7.

To verify that the defect in lysosomal fusion resulting from Rab7 inhibition translated into increased surface expression of 4F2hc, vector control and Rab7 T22N-expressing cells were analyzed for surface 4F2hc levels by flow cytometry (Figure 2C). In the presence of IL-3, Rab7 inhibition did not affect the surface expression of 4F2hc. When IL-3 was withdrawn from control cells, a decrease in 4F2hc surface staining was readily apparent. When Rab7 function was blocked, however, the loss of surface 4F2hc molecules following IL-3 withdrawal was largely prevented.

The degradation of Glut1 was also evaluated. Glut1 is the major glucose transporter expressed in FL5.12 cells. Control cells and cells expressing Rab7 T22N were maintained in or withdrawn from IL-3 for the indicated times and Glut1 content as a percentage of total cell protein evaluated by Western blot (Figure 2D). In the presence of IL-3, interfering with Rab7 function had no effect on Glut1 expression levels. Following growth factor deprivation, Glut1 levels steadily declined in control cells. However, inhibiting Rab7 impeded Glut1 degradation.

Glut1 is heterogeneously glycosylated and does not run as a discrete band in SDS-PAGE gels (Mueckler, 1994). Interestingly, Glut1 from Rab7 T22N-expressing cells includes a population of proteins with decreased mobility in SDS-PAGE. This low-mobility form of Glut1 becomes the dominant form in Rab7 T22N-expressing cells following growth factor withdrawal. Studies of protein trafficking have shown that glycoproteins in the recycling pathway can reenter the TGN and trans-Golgi where their glycosylations can be remodeled (Snider and Rogers, 1985, 1986; Volz et al., 1995). One possibility was that increased recycling of Glut1 in Rab7 T22N-expressing cells led to increased glycosylation due to reiterative trips through the Golgi. To determine whether the observed differences in protein mobility resulted from differences in glycosylation, Glut1 was immunoprecipitated from vector control or Rab7 T22N-expressing cells in the presence and absence of growth factor and digested with endoglycosidase F (Endo F). Removal of glycosylated residues by Endo F treatment produced a single, faster migrating species of Glut1 in both vector control and Rab7 T22N-expressing cells (data not shown). These results indicate that the decreased mobility of Glut1 in Rab7 T22N-expressing cells resulted from differential glycosylation and support the conclusion that interfering with Rab7 function increased the recycling of plasma membrane proteins.

Cells Maintain a Nutrient-Replete Phenotype Despite Growth Factor Withdrawal When Rab7 Is Inhibited

We next wished to determine whether the decreased degradation and increased recycling of nutrient transporters that resulted from blocking Rab7 function prevented the induction of a starvation-like state following growth factor withdrawal. For this reason, 4EBP1 phosphorylation was evaluated by Western blot. The mTOR kinase responds to intracellular amino acids levels, exhibiting increased activity in the presence of amino acids and decreased activity when amino acids are withdrawn (Raught et al., 2001; Rohde et al., 2001; Schmelzle and Hall, 2000). The rate of cap-dependent translation is controlled by mTOR through the regulation of 4EBP1 phosphorylation. mTOR maintains 4EBP1 in the phosphorylated state, rendering it incompetent to bind eIF4E, allowing the formation of the eIF4F complex and thereby increasing the rate of cap-dependent translation. 4EBP1 is phosphorylated at multiple sites and is therefore detected as a series of bands with distinct mobilities in SDS-PAGE. The inhibition of Rab7 had no effect on 4EBP1 phosphorylation in the presence of growth factor (Figure 3A). As expected, 4EBP1 was rapidly dephosphorylated in vector control cells withdrawn from IL-3. When Rab7 activity was blocked, however, 4EBP1 phosphorylation was partially maintained (Figure 3A). 4EBP1 phosphorylation persisted in Rab7 T22N-expressing cells for at least 48 hr after growth factor withdrawal (Figure 3B). Rab7 T22N-supported 4EBP1 phosphorylation was sensitive to treatment with the mTOR inhibitor rapamycin, consistent with the proposal that sustained mTOR activity was responsible for the maintenance of 4EBP1 phosphorylation (data not shown).

It has been suggested that decreased extracellular nutrient uptake following the loss of trophic support leads to a catabolic state in which cells are forced to digest their constituent proteins for energy production (Rathmell et al., 2000, 2001; Vander Heiden et al., 2001). It was possible that blocking Rab7-dependent nutrient transporter degradation would prevent this catabolic atrophy. We therefore evaluated whether Rab7 T22N expression affected the cellular atrophy associated with IL-3 withdrawal. Cell size was measured by flow cytometry. Analysis was restricted to live G1 cells by gating on propidium iodide-negative cells with a 2N DNA content, to eliminate the confounding effects of cell cycle position on cell size. In the presence of IL-3, blocking Rab7 function did not affect cell size (Figure 3C). Following 24 hr of IL-3 withdrawal, a reduction in the size of live G1 cells was observed in all cell lines. However, interfering with Rab7 function resulted in decreased cellular atrophy relative to vector control cells.

Growth factor withdrawal also leads to a decline in the mitochondrial membrane potential (Vander Heiden et al., 1999). One possible explanation for the decrease in $\Delta\Psi m$ is a reduction in the rate of electron transport due to the decreased uptake of oxidizable metabolites. The mitochondrial membrane potential was measured by TMRE fluorescence in vector control and Rab7 T22N-expressing cells in the presence of IL-3 or after growth factor withdrawal (Figure 3D). Blocking Rab7 function did not affect $\Delta\Psi m$ in growth factor-replete cells. As expected, $\Delta\Psi m$ decreased in vector control cells following IL-3 withdrawal. In contrast, cells expressing Rab7 T22N maintained their mitochondrial membrane potential despite IL-3 withdrawal. In summary, increased 4EBP1 phosphorylation, decreased cellular atrophy, and maintenance of the mitochondrial membrane potential following growth factor withdrawal suggest that nutrient uptake is maintained when Rab7-dependent nutrient transporter degradation is prevented.

IL-3 Signaling Is Not Maintained When Rab7 Function Is Inhibited

One possible explanation for the ability of Rab7 T22N to support growth factor-independent nutrient uptake is that, by blocking Rab7 function, the degradation of the ligand-bound IL-3 receptor is inhibited leading to persistent IL-3 signaling despite removal of IL-3 from the medium. To assess whether IL-3 mediated signal transduction is affected by the inhibition of Rab7, we evaluated whether Rab7 T22N expression preserved Pim-1 protein levels or Akt phosphorylation in the absence of IL-3. Pim-1 is an IL-3 responsive protein with a short half-life that disappears rapidly following IL-3 withdrawal (Hoover et al., 2001; Lacronique et al., 2000; Morcinek et al., 2002; Nieborowska-Skorska et al., 2002; Nosaka et al., 1999; Nosaka and Kitamura, 2002). Pim-1 is present in FL5.12 cells as two proteins of 34 and 44 kDa due to alternate translational start sites. Regardless of whether or not Rab7 function was inhibited, the Pim-1 protein was rapidly lost following growth factor deprivation (Figure 4A). The phosphorylation and activation of the serine/threonine kinase Akt is also regulated by IL-3. When IL-3 is withdrawn, Akt phosphorylation at the key regulatory sites Thr308 and Ser473 declines (Figure 4B). Interfering with Rab7 function did not maintain Akt phosphorylation in the absence of IL-3. Thus, the effects of Rab7 T22N expression cannot be attributed to sustained

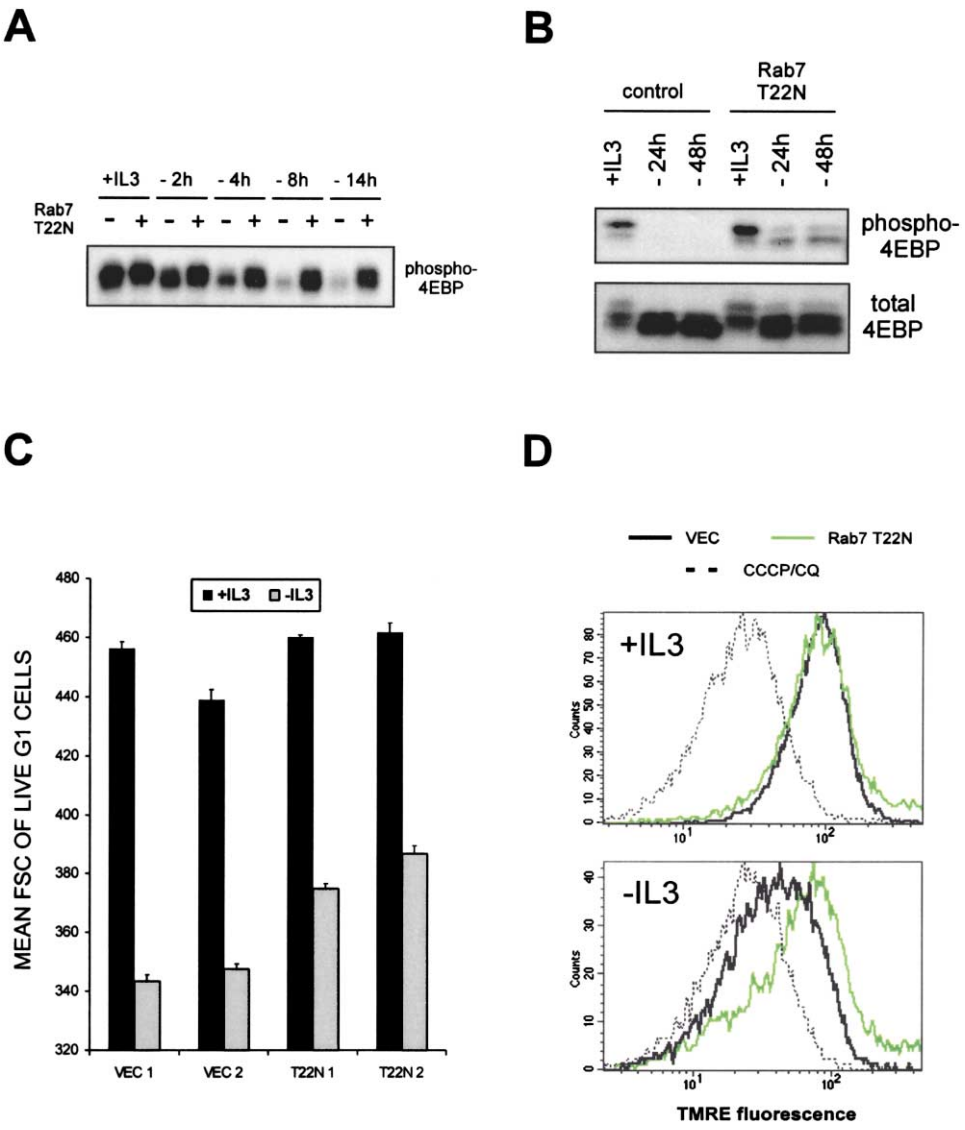


Figure 3. Cells Expressing Dominant-Negative Rab7 Maintain a Nutrient-Replete Phenotype Despite Growth Factor Deprivation

(A) Vector control cells or cells expressing Rab7 T22N were withdrawn from IL-3 for the indicated times, lysed, and 4EBP1 phosphorylation determined by Western blot using a phosphospecific 4EBP1 antibody. Fifty micrograms of total protein was loaded in each lane.

(B) Cells expressing Bcl-X_L or cells also expressing Rab7 T22N were prepared as in (A), but withdrawn from IL-3 for longer intervals. Both phospho- and total 4EBP1 levels were evaluated by Western blot.

(C) Cell lines expressing the indicated transgenes were withdrawn from IL-3 for 24 hr and cell size determined by flow cytometry. Analysis was restricted to live G1 cells by gating on propidium iodide-negative cells with a 2N DNA content. One of three similar experiments is shown. Error bars represent SD; VEC, vector.

(D) Vector control or Rab7 T22N-expressing cells were maintained in or withdrawn from IL-3 for 14 hr as indicated and mitochondrial membrane potential evaluated by TMRE staining and flow cytometry. $\Delta\Psi$m was dissipated by the addition of CCCP and chloroquine (CQ). One of three similar experiments is shown.

Akt activation. Taken together, these results demonstrate that Rab7 T22N expression does not result in persistent IL-3 signaling in the absence of ligand.

Blocking Rab7-Dependent Nutrient Transporter Degradation Confers Growth Factor-Independent, Nutrient-Dependent Cell Survival

If growth factor withdrawal-induced apoptosis results from a state of pseudostarvation induced by nutrient transporter downregulation, then interfering with Rab7 function should increase cell survival following growth factor withdrawal. To test this prediction, the ability of Rab7 T22N to protect FL5.12 cells from IL-3 withdrawal-induced apoptosis was measured (Figure 5A). Vector control cells died with the expected kinetics upon IL-3 withdrawal. When Rab7 function was blocked, however, cells exhibited long-term, IL-3-independent survival. FL5.12 cells expressing similar levels of wild-type Rab7

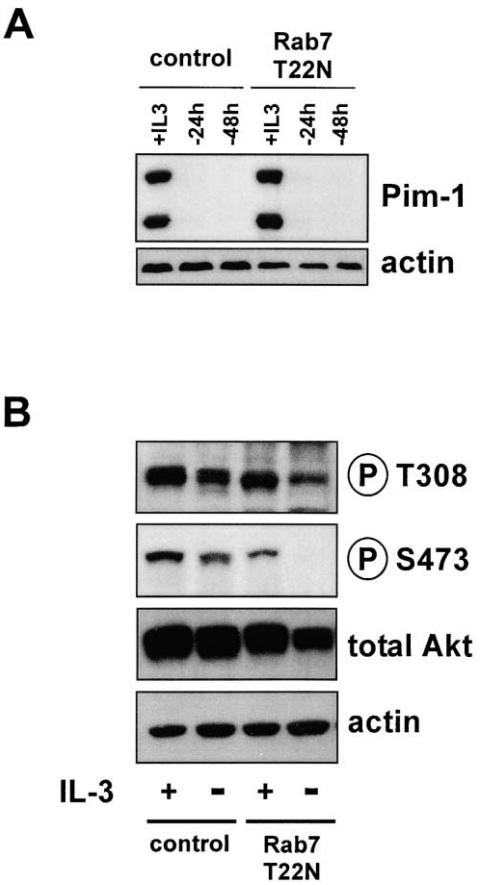


Figure 4. Interfering with Rab7 Activity Does Not Result in Sustained IL-3 Mediated Signaling following Growth Factor Withdrawal

(A) Control Bcl-X_L-expressing cells or cells also expressing Rab7 T22N were maintained in the presence or absence of IL-3 as indicated, lysed, and Pim-1 and actin expression levels evaluated by Western blot. Equal amounts of total cell protein were loaded in each lane.

(B) Cells were prepared as in (A) and evaluated at 24 hr for Akt phosphorylation.

were as susceptible as control cells to IL-3 withdrawal-induced death (data not shown). When IL-3 was resupplied after 5 days of growth factor withdrawal, T22N 1 and T22N 2 cells reentered the cell cycle and proliferated while vector control cells did not (data not shown). Bcl-X_L cells, included as a control, were also protected from apoptosis following IL-3 withdrawal (Figure 5A).

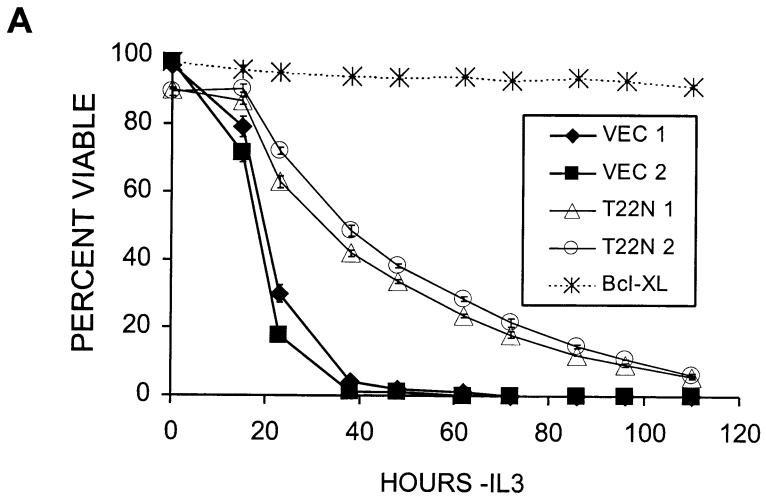
If Rab7 inhibition promotes cell survival by sustaining nutrient uptake, then blocking Rab7 function should not protect cells from apoptosis induced by nutrient limitation. We therefore tested the ability of Rab7 T22N to protect cells from amino acid or glucose withdrawal-induced death (Figure 5B). Amino acid or glucose withdrawal resulted in cell death in vector control cells with similar kinetics to IL-3 withdrawal. Although interfering with Rab7 function protected cells from IL-3 withdrawal, no resistance to amino acid or glucose withdrawal-induced death was observed in Rab7 T22N-expressing cells. In contrast, the antiapoptotic effects of Bcl-X_L did not depend upon the presence of extracellular nutrients.

In order to confirm that the protective effect of Rab7 T22N expression was specific to its interference with Rab7 function, we evaluated the effect on cell survival of reducing Rab7 levels by RNAi. Rab7 levels were decreased in FL5.12 cells following the transient expression of DNA designed to produce a short RNA hairpin targeted against murine Rab7 (Figure 6A). When these cells were withdrawn from IL-3, cell survival was increased (Figure 6B) and cellular atrophy was decreased (Figure 6C), consistent with the results obtained with the dominant-negative construct. Furthermore, cotransfection of wild-type canine Rab7 eliminated the effect of the hairpin targeted against the murine sequence, demonstrating that the increases in cell survival and size resulted from the loss of the endogenous Rab7 protein. The ability of Rab7 T22N expression and reduced Rab7 protein levels to protect from growth factor withdrawal-induced apoptosis strongly suggests that the Rab7-dependent degradation of nutrient transporters contributes to the initiation of growth factor withdrawal-induced apoptosis.

Based on the results obtained with Rab7, we wished to determine whether other proteins in the lysosomal degradation/recycling pathway might also have proapoptotic effects. RILP interacts with Rab7 and may function downstream of this protein by recruiting minus end directed dynein-dynactin containing motor complexes to late endosomes and lysosomes (Cantalupo et al., 2001; Jordens et al., 2001). N-terminally truncated forms of RILP function as dominant-negative proteins and produce fragmentation of the lysosomal compartment similar to dominant-negative Rab7 constructs. An N-terminally truncated form of RILP (C33 RILP) was expressed in FL5.12 cells, resulting in lysosomal fragmentation as evaluated by LysoTracker Red staining (Figure 6D and data not shown). Similar to results obtained in Rab7 T22N-expressing cells, C33 RILP expression resulted in long-term, dose-dependent cell survival in the absence of growth factor (Figure 6E). Both C33 RILP-expressing cell clones were able to proliferate when IL-3 was resupplied following 5 days of growth factor withdrawal, a time point at which no vector control cells could be recovered (data not shown).

Inhibition of Rab7 Function Promotes Cellular Transformation

One step in the process of transformation is escape from the dependence on extrinsic growth factors. As interfering with Rab7 function resulted in a greater ability of cells to maintain growth factor-independent nutrient uptake, Rab7 functions to promote cell-autonomous growth. Thus, the ability of Rab7 T22N expression to promote colony formation by fibroblasts in soft agar was examined. Colony formation in soft agar has been well established to correlate closely with the ability to form tumors in vivo (Freedman and Shin, 1974; Li et al., 1989). Because transformation is a multi-step process, it was unlikely that the inhibition of Rab7 function alone would result in transformation. The p53 protein plays an important role in the suppression of cellular transformation due to its proapoptotic activities (Vousden and Lu, 2002). For this reason, we evaluated whether blocking



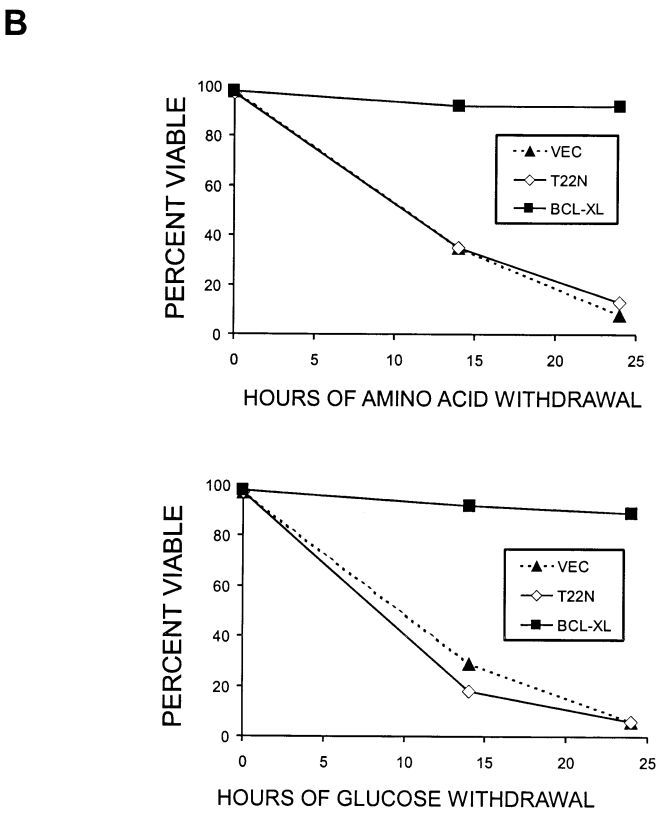


Figure 5. Blocking Rab7 Function Allows IL-3-Independent, Nutrient-Dependent Cell Survival

(A) Cells expressing the indicated transgenes were withdrawn from IL-3 and cell viability measured by propidium iodide exclusion and flow cytometry.

(B) Vector control, Bcl-X_L-expressing, or Rab7 T22N-expressing cells were withdrawn from amino acids or glucose and cell viability measured by propidium iodide exclusion and flow cytometry at the indicated time points. One of at least three independent experiments is shown.

Rab7 activity in p53-deficient cells would allow transformation. Low-passage *p53*$^{-/-}$ MEFs were stably transfected with Rab7 T22N and sorted for high-EGFP expression. When *p53*$^{-/-}$ MEFs expressing empty vector or high levels of Rab7 T22N were examined in soft agar assays, no colonies were formed (data not shown). In addition to resisting apoptosis, transformation requires the ability to enter the cell cycle in the absence of extrinsic signals. The tumor suppressor protein Rb plays an important role in preventing cell-autonomous proliferation (Sherr, 2001). To inactivate Rb, we expressed the adenoviral E1A protein by retroviral transduction. Loss of p53 and inhibition of Rb by E1A was inadequate to transform mouse embryonic fibroblasts (Figure 7). However, when Rab7 function was blocked by Rab7 T22N expression, p53 null E1A-expressing MEFs formed colonies in soft agar.

Discussion

In this report, we show that Rab7 plays a critical role in the downregulation of cell surface nutrient transporters

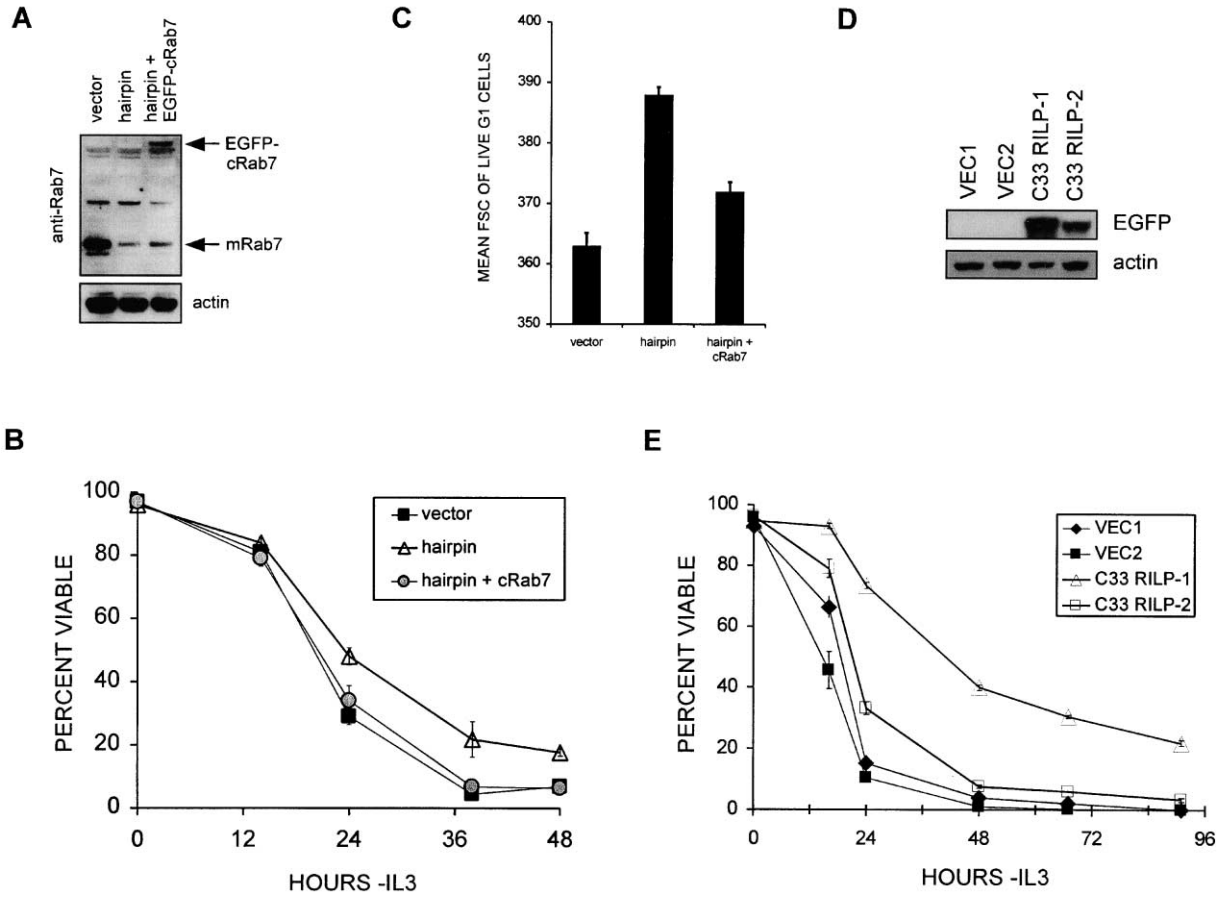


Figure 6. Alternate Means of Inhibiting Rab7 Function Also Support Growth Factor-Independent Cell Survival

(A) FL5.12 cells were transiently transfected with empty vector or a short DNA hairpin directed against murine Rab7 (mRab7) and Rab7 levels evaluated by Western blot. As a control, canine Rab7 (cRab7) was transfected along with the short hairpin.

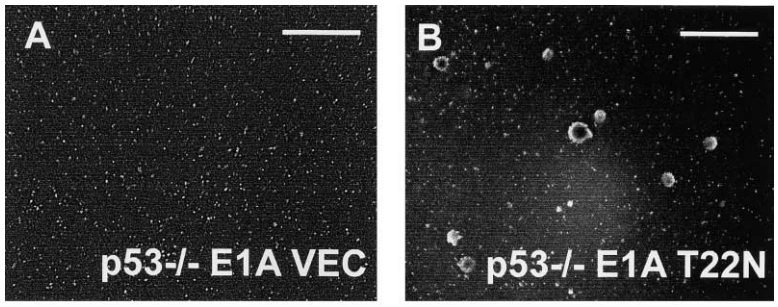
(B and C) A parallel sample of the cells evaluated in (A) was withdrawn from IL-3. Cell survival was measured by PI exclusion and flow cytometry (B) and the mean FCS of live G1 cells determined at the 14 hr time point (C).

(D) FL5.12 cell lines stably expressing empty vector (VEC) or an EGFP-C33 RILP fusion protein were created and evaluated for transgene expression by Western blot.

(E) Vector control and C33 RILP-expressing cells were withdrawn from IL-3 and cell viablility determined by PI exclusion and flow cytometry.

following growth factor withdrawal and thereby contributes to the initiation of apoptosis. When cells are withdrawn from growth factors, their ability to access extracellular nutrients is compromised by the degradation of cell surface nutrient transporter proteins. By interfering with Rab7 function through the expression of a dominant-negative mutant, Rab7 T22N, we blocked the degradation of glucose and amino acid transporter proteins. Blocking Rab7-dependent transporter degradation rendered FL5.12 cells IL-3 independent for nutrient uptake, and many of the atrophic sequelae of growth factor withdrawal were prevented. 4EBP1 phosphorylation was maintained, suggesting that the amino acid-sensitive cell signaling pathways responsible for maintaining 4EBP1 phosphorylation were no longer inactivated. The catabolic cellular atrophy that is most likely a direct consequence of decreased nutrient uptake was also blunted by inhibiting Rab7 function. Finally, $\Delta\Psi m$ was maintained in growth factor withdrawn cells in which Rab7 activity was blocked, indicating that mitochondrial bioenergetics were better supported when cell surface nutrient transporters were preserved. All of these parameters, however, were unaffected by the inhibition of Rab7 function as long as growth factors were present in the medium. Hence, Rab7 activity is dispensable for normal cell growth but is instead involved in arresting cell growth in the absence of growth factor support. Consistent with this model, Rab7 is not an essential gene in yeast, and the Rab7 transcript is induced in FL5.12 cells following IL-3 withdrawal (Wichmann et al., 1992; and C. Fox, personal communication).

Interfering with Rab7 function both through dominant-negative protein expression and by RNAi not only maintained a nutrient-replete phenotype following IL-3 withdrawal but also promoted growth factor-independent, nutrient-dependent cell survival. These results support the conclusion that Rab7-dependent nutrient transporter downregulation plays a central role in the induction of growth factor withdrawal-induced apoptosis. Rab7 has no known function other than the regulation of lysosomal membrane fusion reactions. Thus, it is unlikely that the protection from IL-3 withdrawal-induced



C

	NO. OF COLONIES	
	0.1-0.2 mm	>0.2mm
p53-/- E1A VEC	0	0
	0	0
	1	0
p53-/- E1A T22N	308	13
	310	13
	504	11

Figure 7. Transformation of *p53*$^{-/-}$ MEFs by E1A Is Enhanced by the Expression of Dominant-Negative Rab7

p53$^{-/-}$ MEFs stably expressing empty vector (A) or high levels of Rab7 T22N (B) were transduced with a retrovirus encoding E1A. Infected cells were selected for 2 days in puromycin-containing medium at which point 100,000 cells were plated per 60 mm dish. Plates were photographed after 30 days of incubation. Size bar is 1 mm. (C) Colonies formed on triplicate plates were counted and classified according to colony diameter.

death that results from interfering with Rab7 function is due to a mechanism unrelated to its effects on lysosomal trafficking. Consistent with this, expression of a dominant-negative form of the downstream protein RILP recapitulated the cell survival phenotype observed when Rab7 function was disrupted. Although the decreased growth factor withdrawal-induced protein degradation observed following Rab7 inhibition is not specific to nutrient transporters, the observation that cells were still susceptible to apoptosis induced by nutrient withdrawal demonstrates that increased nutrient access is an important component of the protective effect of blocking Rab7 function. The most likely alternative hypothesis, that persistent growth factor signaling was maintained when Rab7 was inhibited due to recycling of the IL-3-bound IL-3 receptor, was ruled out. Thus, our results are most consistent with a model in which Rab7-dependent nutrient transporter degradation initiates apoptosis following growth factor withdrawal and thereby prevents cell-autonomous growth by mammalian cells.

Our results have implications for oncogenic progression. Full transformation requires that cells suppress cell-intrinsic apoptotic pathways and proliferate in the absence of extrinsic signals. Therefore, neoplastic growth depends on the inactivation of the p53 and Rb tumor suppressor proteins that induce apoptosis and prevent cells from exiting the G1 phase of the cell cycle, respectively, in the absence of growth factors (Sherr, 2001; Vousden and Lu, 2002). However, p53 deficiency in combination with Rb inactivation by E1A was insufficient to promote colony formation by MEFs in soft agar (Figure 7) or to allow tumor formation in nude mice (Degenhardt et al., 2002). Thus, the ability to resist apoptosis and to enter the cell cycle is not sufficient for transformation. As the inhibition of Rab7-dependent nutrient transporter degradation in p53 null, E1A-expressing cells did result in colony formation in soft agar, the acquisition of a mechanism for cell-autonomous nutrient uptake may be a critical step in transformation.

Several proteins involved in the regulation of endocytosis have been linked to cancer. TSC2/tuberin, a gene deleted in the tumor-prone tuberous sclerosis syndrome, is reported to be a GAP for Rab5, a protein that regulates endosomal fusion reactions that occur membrane proximal to those controlled by Rab7 (Xiao et al., 1997). However, unlike Rab7 T22N, expression of a dominant-negative Rab5 construct did not promote FL5.12 cell survival upon IL-3 withdrawal (data not shown). This result is not inconsistent with our model. In order to support nutrient uptake, transporters must be expressed on the cell surface. Inhibiting transporter degradation at a postendocytic step would not be predicted to increase cell survival unless transporter proteins are also shunted into a recycling pathway and reexpressed on the cell surface. While disrupting Rab7 function results in efficient nutrient transporter recycling, interfering with Rab5 function does not appear to support a sufficient level of nutrient transporter surface expression to maintain growth factor-independent cell survival.

Mutations in several endocytic proteins promote cellular transformation by maintaining growth factor signaling in the absence of ligand. Thus far, Rab7 T22N and Rab7 RNAi are unique in their ability to support cell survival without preserving growth factor signaling. Based on the results presented here, other proteins involved in endocytosis and linked to cancer development should be evaluated for their abililty to support growth factor-independent nutrient transporter expression. For example, chromosomal translocations involving three different proteins involved in endocytosis are associated with hematopoetic malignancies (Floyd and De Camilli, 1998), and overexpression of eps15, the murine ortholog of one of these fusion partners, promotes colony formation by NIH 3T3 cells in soft agar. It is likely that some of these proteins promote transformation by maintaining growth factor signaling pathways. However, in light of our findings with Rab7, the ability to support nutrient

transporter expression should be considered as an alternate mechanism by which proteins in the endocytic pathway might promote transformation.

The human Rab7 gene is located on chromosome 3 (3q22.1). Deletions, insertions, inversions, and translocations of this region of chromosome 3 have been identified frequently in hematologic malignancies (Jotterand Bellomo et al., 1992; Kashuba et al., 1997; Mitelman et al., 1997; Wieser et al., 2001). Several other tumor types are also characterized by genomic alterations in this region (Bodmer et al., 2002; Edstrom et al., 2000). The identity of the proposed tumor suppressor protein has not yet been identified, although Rab7 has been shown to be affected in at least one chromosomal rearrangement of this region (Kashuba et al., 1997). Analysis of primary human tumor cells will be important to clarify whether Rab7 activity is decreased in naturally occurring tumors or whether other means of supporting nutrient uptake are more commonly utilized by cancer cells. Once the mechanisms by which primary tumor cells maintain nutrient uptake are identified, these pathways will represent novel, unexplored potential targets for chemotherapeutic intervention. As angiogenesis inhibitors work on the principle of nutrient limitation, it is possible that drugs that stimulate transporter internalization and degradation would be similarly effective.

Experimental Procedures

Materials and Constructs

Antibodies were purchased from Santa Cruz Biotechnologies (Rab7, Pim-1, and actin); Clontech (EGFP); Cell Signaling Technologies (Akt, phospho-Akt Ser473, phospho-4EBP, 4EBP, and anti-rabbit HRP); BD Pharmingen (4F2hc and Lamp-1); Research Diagnostics, Inc (Glut1); and Affinity Bioreagents (phospho-Akt Thr308). The sheep serum used to immunoprecipitate Glut1 was generously provided by Dr. Morrie Birnbaum (University of Pennsylvania). Propidium iodide, Hoechst 33342, and Alexa594 anti-rat secondary were from Molecular Probes. The bicinchoninic acid protein assay kit (Pierce) was used to determine total protein in cell lysates. EGFP-Rab7 (canine), EGFP-Rab7 T22N (canine), and EGFP-C33 RILP (human) were obtained from Dr. Cecilia Bucci (Universita di Napoli, Italy) and cloned into the EF6/V5-HIS mammalian expression vector (Invitrogen). Rab5 and Rab5 S34N were generously provided by Dr. Phil Stahl (Washington University, St. Louis).

The pKD vector used to express short hairpins for RNAi was derived from pBabe-Puro by deleting a portion of the 3′ LTR. The human U6 RNA polymerase III promoter (−265 to −1) was PCR amplified from HeLa genomic DNA and TA cloned into pEF6/V5-HIS generating pEF6-hU6. To generate short hairpin RNAs (shRNA) for RNA interference, a PCR-based strategy was employed using pEF6-hU6 as a template and primers containing the DNA hairpin sequence as previously described (Paddison and Hannon, 2002). The resultant PCR product (330 bp) was TOPO-TA cloned into pEF6/V5-HIS, hairpin sequence in the correct orientation excised with EcoRV and BamHI and ligated into the SnaBI and BamHI sites of pKD. The Rab7 hairpin sequence (sense) is 5′GGG GGA CTC TGG TGT TTG GAA A 3′.

Cell Culture

FL5.12 cells were maintained in RPMI (Gibco) supplemented with 10% FCS (Gemini), 8% WEHI-conditioned medium, 10 mM HEPES, 55 μM β-mercaptoethanol, antibiotics, and L-glutamine. Glucose or amino acid-free RPMI was prepared from chemical components and supplemented with vitamins (Gibco) and dialyzed FCS (Gibco).

Fluorescence Microscopy

FL5.12 cells were fixed for 10 min at RT in 1% paraformaldehyde in PBS, washed in PBS containing 2% FCS and 0.03% saponin, and then incubated sequentially with primary and secondary antibodies for 1 hr at RT in wash buffer containing 0.3% saponin and 10% FCS. Live cells were stained in media containing LysoTracker Red (Molecular Probes) at 50 nM for 30 min at 37°C. Cells were examined using a Nikon E800 fluorescence microscope equipped with a CCD camera or with a Biorad ES confocal microscope. Images were analyzed using the Metamorph software package (epifluorescence) or with LaserSharp (confocal).

Flow Cytometry

For evaluation of 4F2hc surface expression, 500,000 cells were washed with wash buffer (PBS containing 2% FCS and 0.05% sodium azide) and then incubated on ice for 15 min with 5 μg of mouse immunoglobulin (Jackson Immunoresearch) to block Fc receptors. Primary antibody (rat anti-mouse 4F2hc) was added at a 1:1000 dilution in the presence of mouse immunoglobulin, and cells were incubated for 30 min on ice. After two washes with cold buffer, secondary antibody (donkey anti-rat Ig preabsorbed against mouse immunoglobulin, Jackson Immunoresearch) coupled to R-phycoerytherin was added for 30 min. Cells were analyzed on a Becton Dickinson FACS Calibur flow cytometer. For measurements of cell viability and size, cells were incubated with 10 μg/ml of both propidium iodide and Hoechst 33342 for 30 min at 37°C and then analyzed on a Becton Dickinson LSR flow cytometer. Mitochondrial membrane potential was evaluated by incubating 500,000 cells with 10 nM TMRE (Molecular Probes) for 30 min at 37°C prior to analysis using the LSR cytometer. CCCP (50 μM) and chloroquine (4 μg/ml) were added to duplicate tubes to determine background and confirm that TMRE fluorescence was lost upon the dissipation of $\Delta\Psi m$.

Western Blotting

Cells were washed with PBS and lysed in RIPA-containing protease (Complete, Roche) and phosphatase (Phosphatase Inhibitor Cocktail Set 1, Calbiochem) inhibitors. Lysates were cleared by centrifugation and equal amounts of total cell protein were loaded onto Tris-glycine SDS PAGE gels (Invitrogen). Proteins were transferred to nitrocellulose, the membrane blocked with BLOTTO (5% nonfat dry milk and 0.1% Tween-20 in PBS), and incubated with the indicated antibodies prior to probing with ECL (Amersham Pharmacia). A modified sample buffer (250 mM Tris, 5% SDS, 8 M urea, 0.16 M DTT for 2.5X) and incubation at 37°C for 15 min were used when lysates were to be probed for Glut1 to avoid Glut1 aggregation. Glut1 levels were quanitified using the Scion Image software.

Endoglycosidase Digestion

Glut1 was immunoprecipitated from 100 μg of total cell lysate. Immunoprecipitates were resuspended in denaturing buffer and incubated at 37°C for 10 min after which DTT and NP-40 were added per the manufacturers instructions. One unit of Endo F was added and reactions incubated at 37°C for 1 hr; control reactions were prepared in the same manner but incubated without enzyme. Reactions were stopped by the addition of modified sample buffer and changes in glycosylation pattern monitored by Western blot.

Soft Agar Assays

p53$^{-/-}$ MEFs were stably transfected with EGFP-Rab7 T22N and then sorted on a MoFlow Cytomation cell sorter to obtain a population of cells with elevated EGFP fluorescence. Cells were transduced with an MLV-based retroviral vector encoding E1A and then selected in 1 mg/ml puromycin for 2 to 3 days prior to plating. One hundred thousand live cells were plated in 60 mm dishes in complete medium containing 0.38% Noble agar (Difco) layered over a base of 0.6% agar in medium. Plates were incubated for 21 to 30 days at 37°C in a humidified CO_2 incubator, at which point colonies were counted and photographed.

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References

Bodmer, D., Janssen, I., Jonkers, Y., van den Berg, E., Dijkhuizen, T., Debiec-Rychter, M., Schoenmakers, E., and van Kessel, A.G. (2002). Molecular cytogenetic analysis of clustered sporadic and familial renal cell carcinoma-associated 3q13 approximately q22 breakpoints. Cancer Genet. Cytogenet. *136*, 95–100.

Bucci, C., Thomsen, P., Nicoziani, P., McCarthy, J., and van Deurs, B. (2000). Rab7: a key to lysosome biogenesis. Mol. Biol. Cell *11*, 467–480.

Cantalupo, G., Alifano, P., Roberti, V., Bruni, C.B., and Bucci, C. (2001). Rab-interacting lysosomal protein (RILP): the Rab7 effector required for transport to lysosomes. EMBO J. *20*, 683–693.

Cantley, L.C., and Neel, B.G. (1999). New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc. Natl. Acad. Sci. USA *96*, 4240–4245.

Conlon, I., and Raff, M. (1999). Size control in animal development. Cell *96*, 234–244.

Degenhardt, K., Chen, G., Lindsten, T., and White, E. (2002). BAX and BAK mediate p53-independent suppression of tumorigenesis. Cancer Cell *2*, 193–203.

Deves, R., and Boyd, C.A. (2000). Surface antigen CD98(4F2): not a single membrane protein, but a family of proteins with multiple functions. J. Membr. Biol. *173*, 165–177.

Edinger, A.L., and Thompson, C.B. (2002). Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake. Mol. Biol. Cell *13*, 2276–2288.

Edstrom, E., Mahlamaki, E., Nord, B., Kjellman, M., Karhu, R., Hoog, A., Goncharov, N., Teh, B.T., Backdahl, M., and Larsson, C. (2000). Comparative genomic hybridization reveals frequent losses of chromosomes 1p and 3q in pheochromocytomas and abdominal paragangliomas, suggesting a common genetic etiology. Am. J. Pathol. *156*, 651–659.

Feng, Y., Press, B., and Wandinger-Ness, A. (1995). Rab 7: an important regulator of late endocytic membrane traffic. J. Cell Biol. *131*, 1435–1452.

Floyd, S., and De Camilli, P. (1998). Endocytosis proteins and cancer: a potential link? Trends Cell Biol. *8*, 299–301.

Freedman, V.H., and Shin, S.I. (1974). Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. Cell *3*, 355–359.

Gottlob, K., Majewski, N., Kennedy, S., Kandel, E., Robey, R.B., and Hay, N. (2001). Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. Genes Dev. *15*, 1406–1418.

Hoover, R.R., Gerlach, M.J., Koh, E.Y., and Daley, G.Q. (2001). Cooperative and redundant effects of STAT5 and Ras signaling in BCR/ABL transformed hematopoietic cells. Oncogene *20*, 5826–5835.

Jordens, I., Fernandez-Borja, M., Marsman, M., Dusseljee, S., Janssen, L., Calafat, J., Janssen, H., Wubbolts, R., and Neefjes, J. (2001). The Rab7 effector protein RILP controls lysosomal transport by inducing the recruitment of dynein-dynactin motors. Curr. Biol. *11*, 1680–1685.

Jotterand Bellomo, M., Parlier, V., Muhlematter, D., Grob, J.P., and Beris, P. (1992). Three new cases of chromosome 3 rearrangement in bands q21 and q26 with abnormal thrombopoiesis bring further evidence to the existence of a 3q21q26 syndrome. Cancer Genet. Cytogenet. *59*, 138–160.

Kan, O., Baldwin, S.A., and Whetton, A.D. (1994). Apoptosis is regulated by the rate of glucose transport in an interleukin 3 dependent cell line. J. Exp. Med. *180*, 917–923.

Kashuba, V.I., Gizatullin, R.Z., Protopopov, A.I., Allikmets, R., Korolev, S., Li, J., Boldog, F., Tory, K., Zabarovska, V., Marcsek, Z., et al. (1997). NotI linking/jumping clones of human chromosome 3: mapping of the TFRC, RAB7 and HAUSP genes to regions rearranged in leukemia and deleted in solid tumors. FEBS Lett. *419*, 181–185.

Lacronique, V., Boureux, A., Monni, R., Dumon, S., Mauchauffe, M., Mayeux, P., Gouilleux, F., Berger, R., Gisselbrecht, S., Ghysdael, J., et al. (2000). Transforming properties of chimeric TEL-JAK proteins in Ba/F3 cells. Blood *95*, 2076–2083.

Li, L., Price, J.E., Fan, D., Zhang, R.D., Bucana, C.D., and Fidler, I.J. (1989). Correlation of growth capacity of human tumor cells in hard agarose with their in vivo proliferative capacity at specific metastatic sites. J. Natl. Cancer Inst. *81*, 1406–1412.

Mellman, I. (1996). Endocytosis and molecular sorting. Annu. Rev. Cell Dev. Biol. *12*, 575–625.

Mitelman, F., Mertens, F., and Johansson, B. (1997). A breakpoint map of recurrent chromosomal rearrangements in human neoplasia. Nat. Genet. *15 Spec No*, 417–474.

Morcinek, J.C., Weisser, C., Geissinger, E., Schartl, M., and Wellbrock, C. (2002). Activation of STAT5 triggers proliferation and contributes to anti-apoptotic signalling mediated by the oncogenic Xmrk kinase. Oncogene *21*, 1668–1678.

Mueckler, M. (1994). Facilitative glucose transporters. Eur. J. Biochem. *219*, 713–725.

Nieborowska-Skorska, M., Hoser, G., Kossev, P., Wasik, M.A., and Skorski, T. (2002). Complementary functions of the antiapoptotic protein A1 and serine/threonine kinase pim-1 in the BCR/ABL-mediated leukemogenesis. Blood *99*, 4531–4539.

Nosaka, T., and Kitamura, T. (2002). Pim-1 expression is sufficient to induce cytokine independence in murine hematopoietic cells, but is dispensable for BCR-ABL-mediated transformation. Exp. Hematol. *30*, 697–702.

Nosaka, T., Kawashima, T., Misawa, K., Ikuta, K., Mui, A.L., and Kitamura, T. (1999). STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells. EMBO J. *18*, 4754–4765.

Paddison, P.J., and Hannon, G.J. (2002). RNA interference: the new somatic cell genetics? Cancer Cell *2*, 17–23.

Palacin, M., Estevez, R., Bertran, J., and Zorzano, A. (1998). Molecular biology of mammalian plasma membrane amino acid transporters. Physiol. Rev. *78*, 969–1054.

Plas, D.R., Talapatra, S., Edinger, A.L., Rathmell, J.C., and Thompson, C.B. (2001). Akt and Bcl-xL promote growth factor-independent survival through distinct effects on mitochondrial physiology. J. Biol. Chem. *276*, 12041–12048.

Raff, M. (1992). Social controls on cell survival and death. Nature *356*, 397–400.

Rathmell, J.C., Vander Heiden, M.G., Harris, M.H., Frauwirth, K.A., and Thompson, C.B. (2000). In the absence of extrinsic signals, nutrient utilization by lymphocytes is insufficient to maintain either cell size or viability. Mol. Cell *6*, 683–692.

Rathmell, J.C., Farkash, E.A., Gao, W., and Thompson, C.B. (2001). IL-7 enhances the survival and maintains the size of naive T cells. J. Immunol. *167*, 6869–6876.

Raught, B., Gingras, A.C., and Sonenberg, N. (2001). The target of rapamycin (TOR) proteins. Proc. Natl. Acad. Sci. USA *98*, 7037–7044.

Rohde, J., Heitman, J., and Cardenas, M.E. (2001). The TOR kinases link nutrient sensing to cell growth. J. Biol. Chem. *276*, 9583–9586.

Schmelzle, T., and Hall, M.N. (2000). TOR, a central controller of cell growth. Cell *103*, 253–262.

Sherr, C.J. (2001). The INK4a/ARF network in tumour suppression. Nat. Rev. Mol. Cell Biol. *2*, 731–737.

Snider, M.D., and Rogers, O.C. (1985). Intracellular movement of

cell surface receptors after endocytosis: resialylation of asialotransferrin receptor in human erythroleukemia cells. J. Cell Biol. *100*, 826–834.

Snider, M.D., and Rogers, O.C. (1986). Membrane traffic in animal cells: cellular glycoproteins return to the site of Golgi mannosidase I. J. Cell Biol. *103*, 265–275.

Vander Heiden, M.G., Chandel, N.S., Williamson, E.K., Schumacker, P.T., and Thompson, C.B. (1997). Bcl-X_L regulates the membrane potential and volume homeostasis of mitochondria. Cell *91*, 627–637.

Vander Heiden, M.G., Chandel, N.S., Schumacker, P.T., and Thompson, C.B. (1999). Bcl-X_L prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. Mol. Cell *3*, 159–167.

Vander Heiden, M.G., Plas, D.R., Rathmell, J.C., Fox, C.J., Harris, M.H., and Thompson, C.B. (2001). Growth factors can influence cell growth and survival through effects on glucose metabolism. Mol. Cell. Biol. *21*, 5899–5912.

Vitelli, R., Santillo, M., Lattero, D., Chiariello, M., Bifulco, M., Bruni, C.B., and Bucci, C. (1997). Role of the small GTPase Rab7 in the late endocytic pathway. J. Biol. Chem. *272*, 4391–4397.

Volz, B., Orberger, G., Porwoll, S., Hauri, H.P., and Tauber, R. (1995). Selective reentry of recycling cell surface glycoproteins to the biosynthetic pathway in human hepatocarcinoma HepG2 cells. J. Cell Biol. *130*, 537–551.

Vousden, K.H., and Lu, X. (2002). Live or let die: the cell's response to p53. Nat. Rev. Cancer *2*, 594–604.

Whetton, A.D., Bazill, G.W., and Dexter, T.M. (1984). Haemopoietic cell growth factor mediates cell survival via its action on glucose transport. EMBO J. *3*, 409–413.

Wichmann, H., Hengst, L., and Gallwitz, D. (1992). Endocytosis in yeast: evidence for the involvement of a small GTP-binding protein (Ypt7p). Cell *71*, 1131–1142.

Wieser, R., Schreiner, U., Pirc-Danoewinata, H., Aytekin, M., Schmidt, H.H., Rieder, H., and Fonatsch, C. (2001). Interphase fluorescence in situ hybridization assay for the detection of 3q21 rearrangements in myeloid malignancies. Genes Chromosomes Cancer *32*, 373–380.

Xiao, G.H., Shoarinejad, F., Jin, F., Golemis, E.A., and Yeung, R.S. (1997). The tuberous sclerosis 2 gene product, tuberin, functions as a Rab5 GTPase activating protein (GAP) in modulating endocytosis. J. Biol. Chem. *272*, 6097–6100.